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The use of semi-quantitative tests at Cesarean section delivery for the differentiation of canine fetal fluids from maternal urine based on biochemical characteristics

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Abstract

In dogs, there is no diagnostic test to identify and differentiate fetal fluids from maternal urine in the event that a clear-yellowish vulvar discharge is observed pre-whelping. The objective of this study was to find a test that could easily and accurately identify rupture of the fetal membranes preceding parturition. Maternal urine, as well as amniotic fluid (AMF) and allantoic fluid (ALF) from only one fetus per bitch, were collected intraoperatively during Cesarean section. Specific gravity (SG) was analyzed with a refractometer, whereas the presence of leukocytes, protein, glucose, ketones, bilirubin, urobilinogen, nitrite, erythrocyte/hemoglobin (Hb) and the pH were assessed using a urine dipstick (Combur-Test®). Combined calcium and magnesium (Ca/Mg) content were evaluated with the Total Hardness Test. The AmniSure® Test, which detects rupture of fetal membranes in women based on the presence of human placental α-microglobulin-1 was also performed on canine AMF, ALF and urine. Data were analyzed using the Fisher's exact test, Wilcoxon signed rank test and Pearson's correlation. Sensitivity, specificity, positive and negative likelihood ratios (LR) were calculated for parameters with significant difference between urine and both fetal fluids. Maternal urine had higher SG and lower leukocyte, protein, Hb and Ca/Mg content than AMF and ALF. Glucose was more often present in AMF (n=17) and ALF (n=12) than in urine (n=1), while ketone bodies were rarely detected in ALF compared to urine. Bilirubin content was higher in urine and ALF than in AMF. AMF pH was less variable and higher than the pH of ALF or urine. The AmniSure® was negative in all samples tested. Sensitivity and specificity for SG and for the detection of leukocytes, protein, glucose, Hb, Ca/Mg and glucose without ketones in urine and fetal fluids were between 42-100% and 65-100%, respectively. Best positive LR was achieved for the detection of glucose without ketones, and best negative LR for SG ≤ 1.022. In conclusion, the AmniSure® Test, which is used in humans with high diagnostic accuracy, cannot identify AMF and ALF in dogs. Based on our results in 26 dogs undergoing Cesarean section, the presence or absence of fetal fluids could be best determined by a positive glucose test without ketone bodies or by SG > 1.022, respectively.

These tests may serve as additional tools to recognize parturition if clear-yellowish vulvar discharge is present in a term pregnant bitch, but their accuracy and practicability in the clinical setting need to be confirmed.

Keywords: dog, pregnancy, amnion, allantois, parturition

1. Introduction

When a pregnant bitch is presented for leakage of variable amounts of watery, clear or yellowish fluid from the vulva, there is no quick diagnostic test to help determine whether it is composed of fetal allantoic fluid (ALF) and/or amniotic fluid (AMF), or it is maternal urine. Urination may occur more frequently in bitches near the end of gestation due to the increased size of the uterus pressing on the bladder and may sometimes be mistaken with leakage of fetal fluids [1]. Rupture of the allantochorionic membrane (outer fetal membrane) as the fetus enters the birth canal and leakage of the transparent, yellowish allantoic fluid is normal during second stage labor [1, 2]. However, when passage of ALF/AMF occurs before the birth of the first pup without other signs of second stage labor (*i.e.* visible abdominal contractions) it is a sign of dystocia. This may happen in cases of primary uterine inertia, that is, when uterine contractions are completely lacking or too weak for fetal expulsion [1]. In the bitch, uterine inertia accounted for 14% to 49% of dystocia cases [3, 4], or even up to 60% in predisposed breeds like the Boxer [5]. Fast recognition and appropriate therapy are required to avoid neonatal loss. To date, there is no tool for diagnosing rupture of the fetal membranes in dogs, while a rapid test exists for women. The AmniSure® Test (AmniSure GmbH, Wetzlar, Germany) is based on the detection of placental α-microglobulin-1 (PAMG-1), as this protein is present in higher concentrations in AMF compared to other biological fluids [6, 7] and is used as a biomarker [8, 9]. Even small amounts of AMF can accurately be detected in vaginal fluids with the AmniSure® Test, and it appears that artifacts from blood contamination do not significantly interfere with the test's performance [10]. No AMF or ALF-specific proteins have been identified for dogs, and the AmniSure® Test has not been evaluated on canine fetal fluids.

AMF and ALF are produced by various mechanisms: i.e. secretions from the fetus, transudation through the fetal skin before keratinization, or placental, trans- and intramembranous pathways [11-18]. The biochemical characteristics and pregnancy stage-related changes of fetal fluids were already described in women and in several domestic animal species. In cats, detailed information on fetal fluid composition (e.g. electrolytes, proteins, glucose, enzymes of the liver, kidney and pancreas) and its time-dependent evolution was compared to maternal blood but not to maternal urine [19]. In that study, several parameters changed throughout gestation corresponding to the actual degree of fetal development; moreover, changes in fetal fluid composition diverged from those in maternal serum, which underscores the active contribution of the fetus to AMF and ALF production. In dogs, such detailed analysis of fetal fluid composition is missing. Aralla and coworkers [20] found decreased sodium levels in AMF in late- compared to mid-pregnant bitches, while AMF osmolality, urea, total protein and potassium content were similar between the two time points. An immunoprotective role of AMF and ALF was hypothesized after finding lysozyme and immunoglobulin G (IgG) in canine fetal fluids [21]. Another recent study described the presence of insulin-like growth factor 1 (IGF1) and non-esterified fatty acids (NEFA) in the fetal fluids of term pregnant bitches and found that both NEFA and IGF1 concentrations were dependent on the size of the dam [22]. In contrast, AMF glucose, lactate and cortisol levels were not related to breed size, but differences in the values of these parameters were found between puppies of bitches undergoing vaginal parturition or Cesarean section (C-section), and also varied in cases of stillbirth [23]. We hypothesize that the composition of fetal fluids in dogs differs not only from maternal serum but also from urine. The objective of our study was to distinguish fetal AMF and ALF from maternal urine based on biochemical characteristics using semi-quantitative tests at the time of C-section delivery.

2. Material and methods

2.1 Animals

Twenty-six bitches undergoing elective or emergency C-section at the small animal clinic of the Vetsuisse-Faculty, University of Zurich were included in the study. Informed consent was obtained from the owners and the study was approved by the Cantonal Veterinary Authority of Zurich (permit no. 04/2013).

Elective C-sections were carried out in term pregnant dogs with high risk for dystocia. Determination of term gestation was based on a combination of several diagnostic methods. Ovulation was determined by serum progesterone (P4) levels during estrus (P4 ≥ 5 ng/mL at ovulation, [24]). Additionally, and especially if the date of ovulation was unknown, the prepartum P4 profile was followed to estimate the expected time of parturition [25]. Ultrasonography was carried out in all cases to determine gestational age by measuring fetal biparietal diameter, to visualize intestinal peristalsis as an indicator of fetal maturity and to evaluate fetal well-being (heart rate, movements) [26]. C-section was performed only when the bitch was near term (at least 61 days from ovulation or < 2 days before the expected time of parturition) based on the combined results of the methods above.

2.2 Collection and testing of fetal fluids and maternal urine

During C-section, ALF and AMF (1-5 mL) were collected separately from the fetus nearest to the site of hysterotomy. The non-villous allantochorionic membrane was visualized and a small hole was made on it to allow placing a sterile syringe tip into the cavity. The ALF was then aspirated under visual control. Afterwards, the allantochorionic membrane was torn, the fetus in the intact amniotic membrane was visualized and the AMF was aspirated. Only one fetus per bitch was sampled not to prolong time needed to deliver all pups in the litter. This was crucial, as our study population included not only elective but emergency C-sections, where fetal stress may be more pronounced, and the clinical condition of the pups and dams may be compromised compared to elective surgeries. Maternal urine (2-5 mL) was obtained during surgery by cystocentesis after all puppies were delivered. Fluid samples were decanted in a urine collection tube without preservatives and kept at +4 °C until analysis. All tests were carried out within 1 hour of collection.

Specific gravity (SG): Specific gravity of urine, AMF and ALF was analyzed with a manual refractometer (ATAGO Co., Ltd, Tokyo, Japan). A drop of fluid was placed on the refractometer glass and results were read on the appropriate scale (measurement range 1.000-1.050).

Urine colorimetric dipstick test: the Combur-Test® strips (©2010 Roche, Roche Diagnostics Ltd., Rotkreuz, Switzerland) were used on maternal urine and fetal AMF and ALF for comparison among the fluid types. The parameters tested were pH, leukocytes, nitrite, protein, glucose, ketones, urobilinogen (UBG), bilirubin and erythrocyte/hemoglobin (Hb) content. All reaction zones of the test strip were covered with urine, AMF or ALF, and the excess fluid was removed. Results were read after 1 minute by visual inspection. The color reaction of the test pad was compared to the color scale on the label and a value was assigned accordingly. Values of the color scale for each parameter as described on the test label are presented in Table 1. However, when the reaction could not be clearly assigned to one of the neighboring color blocks on the label, we recorded it as 0.5+ above the lower value (e.g. 0.5+, 1.5+, etc.). Negative or normal was recorded as 0.

Calcium (Ca) and magnesium (Mg) concentration: the Total Hardness Test (MQuant™ Total Hardness Test 1.10046.0001, Merck KGaA, Darmstadt, Germany), which was originally developed to measure the hardness of water, was performed on urine, AMF and ALF samples. All five test pads of the strip were covered and then the excess fluid was removed. Results were read after 1 minute by visual inspection and comparing discoloration of the reaction zones to the color scale on the label. Depending on the number of pads on the test strip that turned from green to purple, the following values were available corresponding to a combined Ca and Mg content expressed as $CaCO_3$: negative, 1+ (>90 mg/L), 2+ (>180 mg/L), 3+ (>270 mg/L), 4+ (>360 mg/L) and 5+ (>450 mg/L). When the color change of a reaction zone was not complete but at least half of the reaction pad was discolored, the result was read as 0.5+ added to the number of fully discolored pads (e.g. 0.5+, 1.5+, etc.). When only the edge or less than half of the reaction pad was discolored, it was interpreted as a negative reaction zone.

Placental α-microglobulin-1 (PAMG-1) protein concentration: the AmniSure® Test (AmniSure GmbH, Wetzlar, Germany), which is a one-step immunochromatographic assay, was performed on maternal urine, AMF and ALF. Samples with macroscopic blood contamination were also used, as it seems that blood does not interfere with the test results [10]. The test procedure and reading of results was carried out according to the manufacturer's instructions (www.amnisure.com), except that the sterile swab provided to collect the sample from the vagina in women was dipped for 1 min into the collection tube containing either urine or one of the fetal fluids. When reading the results, the presence of only one line (control line) should indicate a negative test, while two lines correspond to a positive result. If no line is visible, the test is invalid and should be repeated.

2.3 Statistical analysis

To compare the frequency of the Combur-Test® and Total Hardness Test categories between urine, AMF and ALF, the Fisher's exact test using a 2 by 2 contingency table was performed. We classified the Combur-Test® results of leukocytes, glucose, ketone bodies, bilirubin and nitrite into two categories: negative/normal (0) or positive (0.5+ to 3+/4+). The results on protein and erythrocyte/Hb were categorized into low and high: 0 to 1+ (low) and 1.5+ to 3+ (high) for protein, and 0 to 2.5+ (low) and 3+ to 4+ (high) for erythrocytes/Hb. The Total Hardness Test result on Ca/Mg content was also classified into low (0 to 3.5+) and high (4+ to 5+) categories. Specific gravity and pH were analysed by the Wilcoxon signed-rank test with Bonferroni correction in pairwise comparisons. Pearson's correlation was also used for SG. Only samples without macroscopic blood contamination were included in the statistical evaluation. All analyses were performed using SPSS 22.0 software (SPSS Inc., Chicago, IL). Significance was set at $P \leq 0.05$ for the Fisher's exact test and for Pearson's correlation, and at $P \leq 0.0167$ for the Wilcoxon signed-rank test after Bonferroni adjustment.

For parameters which differed significantly between urine and both fetal fluids, sensitivity (SEN), specificity (SPC), positive and negative likelihood ratios (LR) were determined after setting the threshold at the same cut-off value that was used for the categories of the Fisher's exact test, and at ≤ 1.022 for SG. Sensitivity (%) was calculated using the formula SEN = true positive samples/(true positive samples + false negative samples)*100, specificity (%) was determined as SPC = true negative samples/(true negative samples + false positive samples)*100. Positive and negative LR were calculated as SEN/(1-SPC) and (1-SEN)/SPC, respectively. In the equation of the positive LR, 1 was replaced by 0.99 for SPC. If only one of the fetal fluid pairs (AMF or ALF) was available, then that result was used for the calculation. If both AMF and ALF samples were available but the results of the two differed as being true positive or false negative, always the false negative result was used. The same calculations were performed for specific combinations of parameters to increase the value of the diagnostic test.

3. Results

3.1 Animals

Emergency C-section was performed in 17 dogs due to dystocia. Nine bitches underwent elective C-section because of high risk for dystocia due to singleton fetus (n=2), breed predisposition (n=6, English and French Bulldogs, Chihuahua and Maltese breeds) and old age of a primiparous bitch (n=1). Altogether, 100 puppies were delivered by C-section. However, only one fetus per dam was sampled for AMF and ALF adding up to a total of 26 fetuses. Three of those 26 puppies died within two hours after birth despite intensive resuscitation, and a fourth pup had a mild form of anasarca. The other puppies in the study (n=22) were judged healthy, as they were successfully resuscitated, had no gross congenital malformations, nursed colostrum by themselves from the mother soon after C-section, and they kept their body temperature above 35.5 °C in an environmental temperature recommended for their age [27].

Characteristics of the dams and their pups, from which fetal fluids were collected, are presented in Table 2.

3.2 Fluid samples

As stated, we took AMF and ALF from only one fetus per bitch. The amount of urine and AMF collected was sufficient to perform all planned tests in all animals (n=26). However, ALF could only be taken from 24 fetuses, and the amount was insufficient in two of these 24 samples to perform the Combur-Test®. Slight macroscopic blood contamination occurred in the samples of 6 fetuses, *i.e.* in 3 ALF and 3 AMF, so they were excluded from the statistical analysis. Overall, maternal urine and fetal AMF was available from 23 bitch-fetus pairs for statistical comparison. Statistical analysis of SG and the Total Hardness Test was performed on maternal urine and fetal ALF in 21 bitch-fetus pairs, while comparison of the Combur-Test® results of maternal urine and the corresponding fetal ALF was possible in 19 bitch-fetus pairs. AMF and ALF were compared for SG and the Total Hardness Test in 18 fetuses, while the Combur-Test® comparison of AMF and ALF was possible in only 16 fetuses.

3.3 Fetal fluids compared to maternal urine

Specific gravity was lower in AM and AL than in maternal urine ($P < 0.001$ and $P = 0.003$, respectively; Figure 1). There was no correlation between SG of urine and AM or AL ($P \geq 0.190$).

The Combur-Test® was used to compare maternal urine and fetal fluids for the presence of leukocytes, erythrocyte/Hb, protein, pH, glucose, ketones, bilirubin, nitrite and UBG. Leukocytes were detected in several AMF (n=11) and ALF (n=10) samples, but only in one urine sample ($P = 0.001$ and $P < 0.001$, respectively; Figure 2A). Erythrocyte/Hb content was higher in AMF and ALF than in maternal urine ($P < 0.001$ and $P < 0.001$, respectively; Figure 2B). Similarly, high protein content (> 1+) was found in most fetal fluids, but only in one urine sample ($P < 0.001$ and $P < 0.001$, respectively; Figure 2C). The pH was higher in AMF than maternal urine ($P = 0.003$), and was similar between ALF and urine (Figure 2D). Glucose content in both AMF and ALF differed from that in the corresponding maternal urine sample

(P < 0.001 and P < 0.001, respectively; Figure 2E). Seventeen AMF and 12 ALF samples were positive for glucose, while glucosuria was present in only one dog, which was subsequently diagnosed with diabetes mellitus (DM). For ketone bodies, differences were found between maternal urine and ALF (P = 0.027), but not between urine and AMF (P = 0.074; Figure 2F). Ketonuria was detected in 10 of 17 dogs with emergency C-section and 3 of 9 dogs with elective surgery. Nine bitches of small, 3 of medium and 1 of large size breed dogs were affected (Figure 2F). Bilirubin content of maternal urine was different from AMF (P = 0.01) but similar to ALF (P = 0.131; Figure 2G). Only one urine sample tested positive for nitrite, and UBG was normal in all fluids (not shown).

The Total Hardness Test revealed that fetal fluid samples had more often high Ca/Mg content (n=19 of AMF and n=21 of ALF) than maternal urine (n=9) (P = 0.001 and P < 0.001, respectively; Figure 3).

The AmniSure® Test was performed on AMF, ALF and maternal urine from 15 dogs (including 3 AMF and 2 ALF with macroscopic blood contamination). All AmniSure® Tests were valid (*i.e.* the control line was always present) and all samples were negative for PAMG-1. Based on these results, no further testing was carried out on additional animals.

Sensitivity and specificity for SG and for the detection of leukocytes, protein, glucose, Hb, Ca/Mg and glucose without ketones in urine and fetal fluids was between 42-100% and 65-100%, respectively (Table 3). Best likelihood ratios were found for SG and for the combination of glucose without ketone bodies (Table 3).

3.4 Amniotic fluid compared to allantoic fluid

Specific gravity of AMF was lower than ALF (P < 0.001; Figure 1) and there was no correlation between the two (P = 0.292). There were significant differences between AMF and ALF in pH (Figure 2C), erythrocyte/Hb (Figure 2G) and bilirubin (Figure 2F) (P = 0.003, P = 0.035 and P < 0.001, respectively), while all other parameters of the Combur-Test® were similar ($P \geq 0.516$). The Total Hardness Test showed no difference between the two fluid types (Figure 3).

4. Discussion

Using rapid semi-quantitative tests we determined that fetal fluids have a SG between 1.005 and 1.022, a pH of 5-9, and may contain leukocytes, protein, glucose, ketone bodies, erythrocyte/Hb and Ca/Mg. However, more interesting is that we found differences in biochemical composition between fetal fluids and maternal urine, which may help to recognize rupture of the fetal membranes in dogs. While in humans a non-invasive test based on the detection of amniotic fluid PAMG-1 can be used for the clinical diagnosis of premature fetal membrane rupture [8, 9], this test was not diagnostic in our study in dogs. The AmniSure® Test was negative not only in urine but in all fetal fluid samples regardless of macroscopic blood contamination. Therefore, we speculate that in dogs there is either no protein in fetal fluids homologue to the human PAMG-1 or, more likely, that the antibody used in the human assay does not cross-react with the canine protein. These assumptions require further investigation.

Although we did not find a single test parameter which is unique for fetal fluids in dogs, based on the best negative likelihood ratio, a SG > 1.022 is a clear indicator of maternal urine. However, lower values can also be found in bitches with hypo-, isosthenuria and minimally concentrated urine (SG <1.008, 1.008-1.012 and 1.013-1.030, respectively) [28] and, therefore, are not diagnostic. The high variability in urine SG observed in our study may have been the consequence of intravenous fluid administration before and during surgery. Interestingly, the range of SG values in AMF and ALF was less variable, which suggests that their production is tightly regulated through various mechanisms [11-18, 20] and does not respond so quickly to sudden changes in maternal fluid homeostasis. Because all urine samples were collected during surgery after the bitches had received intravenous fluid infusion, the overlap in SG between maternal urine and AMF or ALF may have been greater in this study than it would be during a natural whelping when intravenous fluids are not administered.

Whereas high SG could be used to rule out the presence of fetal fluids, the presence of glucose was highly suspicious for AMF and ALF, as glucosuria should not occur in healthy adult dogs [28]. Glucose in the absence of ketone bodies in the fluid samples further increased the likelihood of positively identifying fetal fluids. If we used that as a marker for AMF and ALF in our data set, all urine samples would have been correctly ruled out, but fetal fluids would have been identified in only 16 dogs. While glucose measured with the Combur-Test® was detected in several AMF and ALF samples, it was present in only one maternal urine sample. This bitch's urine was also positive for ketones. We confirmed that she had diabetes mellitus (DM), which is very uncommon despite the substantial decrease in whole body insulin sensitivity in late canine pregnancy [29-31]. Ketonuria without glucosuria, on the other hand, is common in pregnant dogs due to the pregnancy toxemia and/or anorexia that may precede parturition [31, 32]. This was also observed in 12 of the 26 bitches analyzed, especially in small size dogs. In some cases, ketonuria was also detected together with a positive ketone test in fetal fluids, which is consistent with increased maternal and/or fetal fat mobilization and subsequent ketone production in the affected dogs.

Protein content in the fetal fluids measured with the Combur®-Test was higher than in maternal urine. This is not surprising, as normal urine in dogs contains no or only small amounts of protein [28]. Proteinuria is normal in canine neonates due to the immaturity of the kidneys [27] and thus fetal urine may contribute to the increased protein levels of AMF and ALF. The source of the higher Hb content in the fetal fluids compared to urine might have been the marginal hematoma (hemophagous region) of the placental girdle. Blood pooled in these areas may serve as an iron source for the fetus [33-35]. However, contamination with microscopic amounts of blood during sample collection also contributing to increased Hb and protein levels cannot be completely ruled out. It may also explain the presence of leukocytes in the fetal fluids, although finding them in limited numbers may be normal. Accordingly, in a recent study in mares, most AMF collected at birth also contained a small number of polymorphonuclear leukocytes [36]. Other components of the immune system e.g. lysozyme and IgG have already been identified in the fetal fluids of dogs [21] supporting the role of AMF and ALF in the immunoprotection of the fetus.

In our study, AMF pH was similar among dogs, while the pH of ALF and especially urine varied considerably. AMF pH was also higher than that in ALF, which is comparable to the findings in sheep pregnancy during the last trimester [14]. The higher bilirubin levels we found in canine maternal urine and ALF compared to AMF can be explained by the excretion of bilirubin in the urine of the dam [28, 37] and the fetus, respectively, and perhaps by the conversion of uteroverdin of the marginal placental hematoma into bilirubin contributing to higher levels in ALF.

Using the Total Hardness Test we found higher Ca and/or Mg content in fetal AMF and ALF than in maternal urine, however we were unable to differentiate between the two minerals due to methodology. Their higher content in these fluid compartments may suggest that they could serve as reserve pools for Mg and Ca for the developing canine fetus. Calcium is necessary for bone mineralization, muscle contractions, movements as well as cell signaling and enzymatic reactions within the fetus [38].

Intentionally, we used simple tests accepting subjectivity and limitations of interpretation compared to quantitative laboratory analysis, as they should be practical for breeders and veterinarians in an emergency situation. Therefore, it should be kept in mind that these semi-quantitative tests, which were not validated for canine fetal fluids, should only be used to differentiate among the fluid types and not to draw conclusions on the exact composition of fetal fluids. Furthermore, the collection of fluids was performed during C-section to ensure their origin and to allow clear demonstration of any differences among them. Therefore, another limitation is that this approach does not take into account the potential influence of vaginal secretions on the test result. To eliminate a possible confounding effect from the mixing of vaginal secretions to the fetal fluids, in the clinical setting, collection of mid-stream vulvar discharge fluid may be recommended. We took AMF and ALF from only one fetus of each bitch, which may be considered a bias factor, as it does not take into account individual differences in a litter. However, we might have compromised puppy health by prolonging birth if all fetuses had been sampled, especially in cases of emergency C-sections, which comprised 65% of all deliveries in this study.

5. Conclusions

To accomplish our goal to find a test that can be easily performed and detect rupture of the fetal membranes, we determined several biochemical parameters in fetal fluids and in maternal urine of dogs. We found that the AmniSure® Test, which is used in humans with high diagnostic accuracy, cannot identify canine AMF or ALF. Based on our results in 26 bitches undergoing C-section, the presence or absence of fetal fluids could be best determined by a positive glucose test without ketone bodies or by SG higher than 1.022, respectively. These tests may serve as additional tools to recognize parturition if clear-yellowish vulvar discharge is present in a term pregnant bitch. However, their accuracy and practicability in the clinical setting still need to be confirmed.

Competing interests

The authors declare that they have not received any financial compensation and have no personal relationships with other people or organizations that could inappropriately bias their work.

Authors' contributions

OB designed the study, contributed to sample collection and analysis, evaluated and interpreted the data, and wrote the manuscript. MR analyzed the samples, evaluated and interpreted the data, and helped draft the manuscript. SK collected the samples, contributed to interpretation of data, and critically revised the manuscript. EM contributed to study design, interpretation of data and carefully revised the manuscript. IMR contributed to study design, sample collection, evaluation and interpretation of data, and critically revised the manuscript. All authors read and approved the final manuscript.

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Table 1

Combur-Test® parameter	Values corresponding to the color scale of the test strip				
Leukocytes (Leu)	negative	1+ (~10-25 Leu/µL)	2+ (~75 Leu/µL)	3+ (~500 Leu/µL)	
Nitrite	negative	positive			
pH	5	6	7	8	9
Protein	negative	1+ (0.3 g/L)	2+ (1 g/L)	3+ (5 g/L)	
Glucose	normal	1+ (2.8 mmol/L)	2+ (5.5 mmol/L)	3+ (17 mmol/L)	4+ (55 mmol/L)
Ketones	negative	1+ (1 mmol/L)	2+ (5 mmol/L)	3+ (15 mmol/L)	
Urobilinogen	normal	1+ (17 µmol/L)	2+ (70 µmol /L)	3+ (140 µmol /L)	4+ (200 µmol /L)
Bilirubin	negative	1+	2+	3+	
Erythrocyte (Ery)	negative	1+ (~5-10 Ery/µL)	2+ (~25 Ery/µL)	3+ (~50 Ery/µL)	4+ (~250 Ery/µL)
Hemoglobin (Hb)	negative	1+ (~10 Ery/µL)	2+ (~25 Ery/µL)	3+ (~50 Ery/µL)	4+ (~250 Ery/µL)

Color scale values of the Combur-Test® are shown for each parameter as indicated on the label.

Table 2

Bitch	N	BW (kg)	Age (years)	Primi-/Pluriparous (N/N)	Elective/ Emergency C-section (N/N)	Litter size	Pup birth weight (g)	Male/Female pup (N/N)
Large/Giant (>30kg)	6	43.1 ± 12.4	5.3 ± 1.8	3/3	2/4	5.3 ± 3.0	463 ± 156	3/3
Medium (10-30kg)	7	19.2 ± 5.4	4.4 ± 2.0	3/4	1/6	3.9 ± 3.4	376 ± 65	5/2
Small (<10 kg)	13	4.8 ± 2.1	2.5 ± 2.0	11/2	6/7	3.4 ± 1.8	153 ± 51 (N=12)	5/7 (N=12)

Body weight (BW), age, parity, type of C-section and litter size of the bitches included in the study (n=26). Birth weight and gender of the puppies from which fetal fluids were collected are shown (available from n=25). Data are presented as mean ± standard deviation. N: number of animals

Table 3

Threshold	TN (N)	FP (N)	TP (N)	FN (N)	Sensitivity (%)	Specificity (%)	Positive LR	Negative LR
SG ≤ 1.022	17	9	26	0	100	65	2.9	0
Leukocyte > 0	25	1	11	15	42	96	11	0.6
Glucose > 0	25	1	18	8	69	96	18	0.3
Protein > 1+	25	1	12	14	46	96	12	0.6
Hb > 2.5+	24	2	22	4	85	92	11	0.2
Ca/Mg > 3.5+	17	9	22	4	85	65	2.4	0.2
Glucose > 0 and no ketone bodies	26	0	16	10	62	100	61.5	0.4

Sensitivity, specificity, positive likelihood ratio and negative likelihood ratio of those parameters, where significant differences between maternal urine and both fetal fluids were found. The threshold for each parameter was set at the same cut-off value that was used in the Fisher's exact test and ≤ 1.022 for SG. TN: true negative, FP: false positive, TP: true positive, FN: false negative, LR: likelihood ratio, N: number of samples; SG: specific gravity; Hb: erythrocyte/hemoglobin; Ca/Mg: combined calcium and magnesium content

Figure captions

Figure 1 Specific gravity of the fetal fluids and maternal urine in each individual animal

Dogs included in the study are presented on the X axis from number 1 to 26. Number 1-6 are the large/giant size (>30 kg) bitches, number 7-13 are the medium (10-30 kg) and 14-26 are the small size (<10 kg) dogs. No. 13 is the bitch with diabetes mellitus. Maternal urine is marked with blue, amniotic fluid (AMF) with red and allantoic fluid (ALF) with green.

Figure 2 Combur-Test® parameters measured in the fetal fluids and in maternal urine in each individual animal

A: Leukocytes; B: Erythrocyte/hemoglobin C: Protein; D: pH; E: Glucose; F: Ketones; G: Bilirubin. Dogs included in the study are presented on the X axis from number 1 to 26. Number 1-6 are the large/giant size (>30 kg) bitches, number 7-13 are the medium (10-30 kg) and 14-26 are the small size (<10 kg) dogs. No. 13 is the bitch with diabetes mellitus. Maternal urine is marked with blue, amniotic fluid (AMF) with red and allantoic fluid (ALF) with green.

Figure 3 Combined calcium and magnesium content of the fetal fluids and maternal urine in each individual animal

Dogs included in the study are presented on the X axis from number 1 to 26. Number 1-6 are the large/giant size (>30 kg) bitches, number 7-13 are the medium (10-30 kg) and 14-26 are the small size (<10 kg) dogs. No. 13 is the bitch with diabetes mellitus. Maternal urine is marked with blue, amniotic fluid (AMF) with red and allantoic fluid (ALF) with green.